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(54) Title: A METHOD OF VACCINE OR TOXOID PREPARATION AND IMMUNIZATION BY COLONIZATION WITH RECOMBINANT MICROORGANISMS

(57) Abstract

A method of vaccine or toxoid preparation and immunization against pathogenic disease. An area of the body is colonized with indigenous microorganisms that have been transformed by recombinant DNA technology to express pathogenic antigens. The colonized transformant microorganisms will replicate and elicit an immune response to the pathogenic antigen, thereby protecting the individual from subsequent exposure to the intact pathogen.

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DESCRIPTION

A Method of Vaccine or Toxoid
Preparation and Immunization by Colonization
With Recombinant Microorganisms

5 Field of the Invention

       This invention relates to a method of vaccine or
toxoid preparation and immunization against pathogenic
diseases by colonizing an area of the body with indigenous
microorganisms that have been transformed by recombinant
10 DNA technology to express pathogenic antigens. More
particularly, this invention relates to a method of
vaccine preparation and immunization against Herpes by
colonizing the genital tract with lactobacillus that has
been transformed by recombinant DNA technology to express
15 the immunologically significant antigens of the Herpes
virus.

Background of the Invention

       A pathogenic microorganism is any microorganism
capable of producing disease, and pathogenicity refers to
the ability of the microorganism to gain entrance to a
20 host and produce physiological or anatomical changes. To
initiate disease, not only must pathogenic microorganisms
enter the body in adequate numbers, but many of them must
enter through a certain route called the "portal of
entry." This mode of entry differs for each individual
25 microorganism depending on its ability to attack certain
cells and tissues. For example, the alimentary tract is
the portal of entry for the typhoid and cholera organisms.
Tuberculosis, diphtheria, and pneumococcus microorganisms
enter through the respiratory tract and may set up infec-
30 tions in the bronchi and lungs. The gonococci, herpes
virus, and some other sexually transmitted microorganisms
generally enter through the urogenital tract from which
they can easily attack the genital organs. Still other

organisms enter through abrasions or openings in the skin and set up local infections, or spread through the body in the circulatory system.

Protective immunity is the ability of a host to 5 prevent or overcome invasion by these pathogenic micro-organisms. Immunity may be acquired as a result of the host's recovery from a pathogenic infection, or may be prophylactically induced by administration of a vaccine or toxoid.

10   Vaccines are suspensions of killed, or living but attenuated microorganisms, or their antigenic portions. Toxoids are detoxified but still antigenically active poisons produced by certain bacteria. Injection of these immunogens (vaccine or toxoid) stimulates an immune 15 response against the specific antigen. Upon subsequent exposure to this same or antigenically related pathogen, the host's immunity assists in defense against the pathogen.

The protection against infection afforded by vaccina-20 tion operates primarily through the humoral arm of the immune response, with the participation of all major classes of immunoglobulin: IgM, IgA and IgG. Circulating neutralizing IgM, IgA and IgG antibodies interrupt or retard the extracellular dissemination of the pathogen. 25 In addition, the roll of cell-mediated immunity appears to have an effect on the type and intensity of the immune response. Local immunity on the other hand, is restricted to the area of pathogen entry and is mediated by local production and release of IgA antibodies. The secretory 30 IgA antibodies function by neutralizing the pathogen before it makes contact with its target cells, thus preventing implantation and formation of infection. IgG and IgM antibodies also appear in secretions but only in low concentrations.

35   In developing and administering vaccines or toxoids, the pathogenesis of each particular disease must be considered, since the portal of entry and location of the

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pathogenic microorganism in the host determine what class
of antibody will provide a protective role.   In viral
respiratory infections, for example, where the focus of
the infection is in the ciliated epithelium of the
5   respiratory tract, virus is released mainly on the mucosa
surface and does not penetrate into the underlying tissue.
As a result, antibodies in the secretions play a major
role in the host's defense whereas circulating antibodies
participate only insofar as they filter through into the
10   secretions.

A major goal in the prevention of infectious diseases
has been the development and use of vaccines or toxoids
for immunization.   Inactivated and attenuated strains of
bacteria and viruses are now widely used for immunization
15   against many diseases including typhoid fever, poliomyeli-
tis, influenza, rabies, measles, and hepatitis type B in
humans.   (Manual of Clinical Laboratory Immunology, 3rd
Ed., Rose, Friedman & Fahey, ASM, D.C., 1986).   For those
bacterial infections where virulence is due in major part
20   to exotoxins such as diphtheria and tetanus, detoxified
but antigenically active toxoids can be used to neutralize
the toxin.   In addition, recent advances in molecular
biology and peptide synthesis have allowed production of
purified viral proteins or synthetic peptides for use in
25   immunoprophylaxis. (Fundamental Virology, Fields & Knipe,
Raven Press, NY, 1986).

In most cases, inactivated virus vaccines are
prepared from virus that is grown in eggs (influenza type
A and B), monkey kidney cell culture (polio virus, types
30   1, 2 and 3) or human diploid fibroblast cell culture
(polio virus, rabies), and then inactivated with formalin.
These vaccines offer the advantage of immunization with
little or no risk of infection but are considerably less
effective than the actual virus infection.

35   Failure to successfully inactivate vaccine virus has
occasionally had serious consequences, as with the for-
malin-inactivated measles virus vaccine.   Initially this

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vaccine prevented measles, but after several years the
vaccinees lost their immunity. When subsequently infected
with measles virus, the vaccinees developed an atypical
illness with accentuated systemic symptoms and pneumonia.

5   It is believed that the formalin used to inactivate the
virus destroyed the antigenicity of the measles F protein,
but did not affect the H-protein. The vaccinees developed
an unbalanced response that included H-protein immunity
but not F-protein immunity. It is also known that inac-

10 tivated influenza virus vaccine appears to lose its
effectiveness after several years, although the basis for
this is not known.

Attenuated vaccines contain viable but weakened
organisms. They work by producing a mild infection which

15 is usually of little danger to the host. The major
advantage of live virus vaccines is their activation of
all phases of the immune system; systemic and local,
immuno-globulin and cell-mediated. Furthermore, immunity
induced by live virus vaccines is generally more durable,

20 more effective and more cross-reactive than that induced
by inactivated vaccine. In addition many live virus
vaccines are easy to administer and are relatively inex-
pensive.

The disadvantages of live attenuated virus vaccines

25 include the potential for contamination with live adven-
titious agents such as other viruses, and the fact that
some live virus vaccines, such as the measles virus,
rubella virus and yellow fever virus vaccines, retain a
low level of residual virulence which may cause mild

30 symptoms of the disease against which the vaccine is
directed. More serious problems include those in which
rare members of the population are particularly vulnerable
to the vaccine strain of the virus, e.g. poliovaccine
virus, resulting in paralysis, or where infection by live

35 vaccine virus occurs in immuno deficient individuals. And
finally, stability is a serious problem with labile
vaccine viruses, and the need for storage and transport of

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some vaccines at low temperature (measles vaccine) has limited their usefulness in some tropical areas where this maintenance is difficult.

Immunity to infection by pathogenic microorganisms
5 depends on the development of an immune response stimulated by antigens associated with each organism. In some instances, as with viruses, antigens present on the surface of the virus play an important role. In other cases, as with diphtheria or tetanus, immunity is a
10 function of specific antibody to the antigenic toxins produced. Therefore, a successful strategy of immunoprophylaxis against pathogenic diseases requires the generation of an immune response to these "protective antigens", i.e. those antigens that stimulate immunity
15 against the intact pathogenic microorganism.

In recent years, the protective surface antigens important in immunity have been identified for a wide range of viruses (Fundamental Virology, Fields & Knipe, Raven Press, N.Y. 1986). With knowledge of the structure
20 or conformation of a particular protective antigen, a synthetic or biosynthetic molecule can be prepared that has the same structure and is capable of provoking antibodies reactive with the intact pathogenic organism.

Two separate approaches have been explored for production of these protective antigens. One involves the production of synthetic peptides which represent immunologically important areas on the surface of the pathogen.
5 The other is recombinant DNA technology which involves splicing a DNA sequence that codes for the pathogenic antigen into a prokaryotic or eukaryotic cell. Subsequently, the cloned pathogen DNA can be expressed in the host cell as pathogen antigen. For example, foot-and-
10 mouth disease virus (FMDV), VP1 capsid protein, hepatitis B virus (HBV) surface antigen, influenza A virus hemagglutinin, rabies virus surface glycoprotein and the gD protein of herpes virus have been expressed in bacteria.

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In addition to the potential hazards of vaccines discussed above, there are many other problems associated with current immunization techniques. Some pathogenic microorganisms themselves possess attributes that create
5   uncertainties for developing satisfactory vaccines. For example, many viruses only produce local infections that tend to shield them from the full play of the host's defense system. Also, the inability to grow hepatitis B virus in tissue culture or in animals other than chimpan-
10 zees, has prevented the development of a conventional live vaccine. (Nature, 302:490-495 (1983)).

The use of protective antigen vaccines is an attractive alternative because it enables circumvention of some of the concerns discussed above that are associated with
15 intact viral immunogens. However, synthesis of relevant protective antigens in bacteria or yeast directed by expression vectors containing cloned DNA copies of the appropriate pathogen genes may not always provide abundant amounts of antigen for use in a vaccine. Many viral
20 antigens (e.g. hepatitis-B virus surface antigen, vesicular stomatitis virus G protein and rabies virus glycoprotein) when expressed in E. coli, are either unstable or lethal to the cell. And attempts to directly express the influenza viral surface glycoprotein hemagglutinin (HA) in
25 large quantities using bacterial promoters have failed. (Proc. Nat. Acad. Sci., 78:5376).

Summary of the Invention
Many indigenous microorganisms abound in and on the human body as well as in animals of other species.
30 Although there is no hard and fast rule which divides these microorganisms into clear-cut categories of harmless, commensal organisms and pathogenic species, microbiologists have attempted to list and categorize them accordingly. Microorganisms that exist in conjunction
35 with the host, resist the host's defense mechanisms but at the same time are unable to invade body tissues. These

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microorganisms are capable of colonizing various areas of
the host and are generally regarded as normal inhabitants.

    Among those microorganisms indigenous to the mouths
of healthy individuals are the staphylococci, micrococci,
5 viridans streptococci, enterococci and lactobacilli.
These organisms exist in the mouth in the interstices
between the teeth, and in and under tooth plaques. The
bacteria commonly present in the nose and throat consist
of still different forms such as pneumococci and strep-
10 tococci, and the bacterial types present in the intestine
are determined to a considerable extent by the type of
food material present which varies with diet and pH.
Although many organisms can be cultured from the normal
female genital tract, the usual aerobic microflora is
15 dominated by the lactobacilli. (Manual of Clinical
Microbiology, 2nd Ed., Lennette, Spaulding and Truant,
eds., American Society for Microbiology, Washington, D.C.
1974).

    We propose a method of immunization against pathogen-
20 ic disease by colonizing an area of the body with these
indigenous microorganisms that have been transformed by
recombinant DNA technology to express protective antigens.
The colonized transformant microorganisms will replicate
at the mucosal surface thereby eliciting an immune respon-
25 se to the protective antigen and protecting the individual
from subsequent exposure to the intact pathogen.

Detailed Description

    Although the method described in my invention may be
used to immunize an individual against any known pathogen,
30 a preferred embodiment is for immunization against the
herpes simplex virus (HSV). The following description
therefore, is mainly directed to immunization against HSV,
but the invention is not intended to be restricted to this
use.

35 Herpes simplex viruses are among the most common
infectious agents of man. The number of new cases of

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herpes infections per year is not known precisely, but
minimally it is estimated to be several hundred thousand
(JAMA 253:1601 (1985)). There are at least two distinct
serotypes of the virus, HSV-1 and HSV-2. Both serotypes
5 cause persistent and latent infections, including recur-
rent cutaneous disease and viral encephalitis.

There have been numerous attempts to develop vaccines
against HSV-2. Concern about producing latent infections
with attenuated live vaccines has shifted attention toward
10 killed and more specifically subunit vaccines. The
principle candidate for these subunit vaccines is HSV
glycoprotein D (HSV gD), the major structural antigen of
the virus responsible for the induction of humoral and
cellular immunity (J. Virol. 41:478 (1982)). However,
15 recent controlled trials of a parenterally administered
HSV gD vaccine has produced somewhat disappointing results
(Abstracts, ICAAC, 1987).

Although most effort has focused on development of
the antigenic components of a vaccine, there has also been
20 some interest in the route of vaccine administration, and
whether immunization at the site of infection might have
an advantage over parenteral administration. In general,
antigens given parenterally are believed less able to
stimulate immunity at the mucosal surfaces (J. Clin.
25 Immuno 7:265 (1987)). Clinical data moreover, suggest
that parenteral immunization against V.cholera may sup-
press the mucosal response to locally applied antigens (J.
Immuno 124:307 (1980)). However, mice immunized in-
travaginally with thymidine kinase negative strain of HSV-
30 2 were found to be protected against an intravaginal
challenge that was lethal to unimmunized control mice.
(J. Virol. 51:747 (1984)).

The lactobacilli are the most common aerobic or-
ganisms found in the vagina of the human. Metchnikoff
35 proposed that the lactobacillus is essential for well
being (ASM News 54:12 (1988)), and Reid et al. found that
the organisms may even protect against urinary tract

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infection. (<u>Infec. Immun</u>. 49:230 (1985)). Additionally,
lactobacilli are found normally in the male urethra (<u>J.
Clin. Microbiol</u>. 6:482 (1977)).

5   I propose a vaccine for immunizing an individual
against HSV by colonizing the female genital tract with an
indigenous strain of lactobacillus that has been trans-
formed by recombinant DNA technology to express HSV
glycoproteins. This technique may also be applicable to
the male by colonizing the urethra.

10   Recent developments of a host vector system for
lactobacilli make it possible to introduce genes into
these bacteria. (<u>FEMS Micro Rev</u>. 46:297-312 (1987)). The
recombinant lactobacillus of my invention contains a
promoter, an alpha-amylase gene, a structural gene coding

15 for the protective antigen, and a marker to establish
whether the insertion of the gene has been successful and
is in the correct reading frame. The amylase portion
results in the secretion of the attached HSV gD, by the
lactobacillus. A beta galactosidase marker is used rather

20 than an antibiotic resistant marker to avoid colonization
and potential spread of an antibiotic resistant strain of
lactobacillus. Alternatively an antibiotic resistant gene
can be used and the antibiotic to which the organism is
resistant used to eliminate existing flora to favor the

25 survival of the colonizing resistant strain.
       The vectors useful for strain construction in lac-
tobacilli have been previously described as efficient
vehicles for cloning in many lactobacillus strains. (<u>FEMS
Microbiol. Revs</u>. 46:297-312 (1987); <u>J. Clin. Microbiol</u>.

30 6:482 (1977); <u>Infec. Dis. Clin. of N. Amer</u>. 2(1):1 (1988);
<u>Trends. Biotechnol</u>. 3:273-275 (1985); <u>Biochimie</u> 70:503-
517 (1988)). The method of the invention involves insert-
ing genes for the glycoproteins of herpes simplex viruses
via a vector into strains of lactobacilli that have been

35 shown to normally be the predominant species in the vagina
(or urethra in the male). The recombinant lactobacilli
are screened in solid media for the HSV gD insert by the

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production of beta galactosidase and then by hybridization
of the nitrocellulose paper lifts with the HSV gD frag-
ment. Positive colonies are picked, cultured, and tested
for expression of HSV gD by fixing cells and incubating
5   them with an anti-HSV gD antibody and then staining them
with fluorescein conjugated immunoglobulin. They are then
mounted on slides and examined by fluorescence microscopy.
In addition, the bacteria or bacterial broth can be tested
for HSV gD in an ELISA system or a polyacrylamide gel and
10   Western blot. In either case, an antibody against HSV gD
may be used in the detection system.

     The recombinant lactobacilli are inoculated into the
vagina. Presence of infection in the colonized animal
model is determined by flushing the vagina at daily
15   intervals with lactobacillus carrying medium (LCM), an
aliquot of which is plated immediately onto an agar plate
using an LCM base (_J. Microbiol._ 48:994 (1984)). After
incubation overnight at 37°C, lifts are made with nitroce-
llulose paper hybridized to a nick-labeled HSV gD probe.
20   The proportion of colonies containing the insert and their
rate of disappearance are then calculated.

     The production of an immune response to the herpes
simplex virus is established by testing vaginal secretions
and serum for the presence of antibody against herpes.
25   This is done by modifying the anti-HSV ELISA which we
described previously. (_J. Clin. Microbiol._ 26:781 (1988)-
). Testing for IgA is done using an anti-alpha conjugate
and anti-mouse rather than anti-human reagents. The
seronegative range is established by testing vaginal
30   secretions and sera of mice prior to inoculation. Testing
is performed starting at one week following colonization
and repeated weakly.

     When there is evidence of an immune response to HSV
gD, the animals are then challenged intravaginally with a
35   dose of herpes virus which has been shown to produce
death, latent infection, or other clinical signs of herpes
vaginitis in unprotected mice. The effectiveness is

studied by comparing mortality, latent virus infection of
spinal ganglia, and virus shedding in unprotected mice and
in mice inoculated with lactobacilli containing the gene
for herpes virus glycoproteins.

5   Example 1
     Preparing the Constructs:
          The structural gene for HSV gD is cloned in a vector
     suitable for transformation of Lactobacilli.   It is
     preferable to start with a vector with a multi-cloning
10   region.   To this is added the promoter and secretion
     leader sequence of the alpha-amylase gene of Bacillus
     amyloliquifaciens in order to direct secretion of HSV gD.
     One skilled in the art will know that other promoter
     sequences that function in Lactobacilli can be substituted
15   for the alpha-amylase promoter sequence.   The HSV gD
     structural gene is then inserted in the correct reading
     frame with the promoter and secretion leader sequence.
          The HSV gD gene is mutagenized and truncated using
     synthetic oligonucleotides, and the new sequence is
20   verified by DNA sequencing.   The truncated form is relega-
     ted into a vector with the appropriate promoter and
     secretion leader sequences.
          One skilled in the art will recognize that this
     forced cloning is designed to be orientation specific and
25   produce in-frame translational fusions of the alpha-
     amylase secretion leader to the HSV gD gene product, so
     that any other available technique may be used.   The
     ligation mixtures are transformed into L. casei by electr-
     oporation as described by Chassy (Trends Biotechnol.
30   3:273-275 (1985)).
          The transformed mixtures are inoculated into Lac-
     tobacillus carrying medium (LCM) and allowed to develop
     for two hours at 37°C.   Portions are spread on LCM plates
     containing 10 μg/ml chloramphenicol (cm), and the plates
35   incubated for two days at 37°C in a Gas-Pack (BBL).
     Colonies (transformants) are streaked onto LCM-Cm plates

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for purification. One skilled in the art will recognize
that alternatively, the colonies can be "lifted" onto
nitro-cellulose and probed by DNA-DNA hybridization for
the presence of HSV gD coding sequences. The HSV gD-
5   containing restriction fragment described above (i.e.
EcoRI to Hind III) is radiolabeled by the random priming
technique and used as a probe for colonies containing the
HSV gD gene.

Cultures of random transformant colonies, or those
10   hybridizing to the HSV gD gene probe, are analyzed for
plasmid content by the standard procedure for Lactobacilli
known in the art. Using agarose gel analysis of restric-
tion enzyme digests of plasmids isolated from transfor-
mants, the screening will ascertain if the HSV gD gene has
15   been inserted in the correct orientation and verify that
the entire coding sequence was inserted. One skilled in
the art will know the unique restriction sites available
to analyze a correct cloning sequence that carries the
alpha-amylase gene fused to the intact HSV gD polypeptide
20   coding sequences.

One skilled in the art will recognize that the
constructs described above can be made in E. coli using
pNZ12 and its derivatives as a shuttle vector, and then
subsequently transferred to L. casei or other predominant
25   strain of lactobacillus in the vaginal flora. Alterna-
tively. pIL253 can be employed for direct cloning in
Lactobacilli.

Example 2
Testing the Constructs for HSV gD Synthesis:
30   Selected clones carrying the desired recombinant
construction described in Example 1 above, are cultivated
in 1 liter LCM-10μg/ml chloramphenicol cultures at 37°C
for 18 hours. Control cultures contain only the vector.
The bacteria are collected by centrifugation and separated
35   from the growth medium. The growth medium is concentrated

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to 10 ml and dialyzed against phosphate buffered saline (PBS).

To determine cell-associated HSV gD, a sample of the bacteria is assayed directly by UV-microscopy and by
5 analysis of the disrupted cell contents. The HSV gD present in the growth medium is also analyzed. These procedures follow:

(1) The bacteria are resuspended in PBS, centrifuged and washed in PBS three times. A small sample of bacteria in
10 the final pellet is placed on a microscope slide and air dried at room temperature. The bacteria are then fixed, incubated with rabbit anti-HSV gD antibody, rinsed with PBS, incubated with fluoscein-conjugated anti-rabbit globulin, rinsed, mounted in alkaline glycerol buffer and
15 examined for fluorescence by fluorescence microscopy.

(2) An aliquot of the washed pellet bacteria above is disrupted in a glass bead mill (Biospec, Barthesville, Okla.) The cell debris and insoluble material are sedimented by centrifugation. The bacterial supernatant will
20 be analyzed as in (3) below.

(3) The bacterial supernatant from (2) and the dialyzed concentrated growth medium are diluted 1:1 with electrophoresis sample buffer. The HSV gD protein is separated on 11% SDS polyacrylamide gels and transferred to nitrocel-
25 lulose membrane by electroblotting at 100 V for 2.5 hours. The membrane is washed in PBS containing 0.3% Tween-20 (PTB), reacted overnight with a solution of PTB containing 4% normal goat serum and rabbit anti-gD, washed with PTB, incubated with biotinylated goat anti-rabbit IgG, washed
30 with PTB, and reacted with streptavidin-horseradish peroxidase, or streptavidin-alkaline phosphatase. Color is developed with 4-chloro-1-naphol (for horseradish peroxidase) or 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/nitroblue tetrazolium chloride (for alkali-
35 ne phosphatase).

The gel lanes are as follows: (1) the concentrated growth medium supernatant from the transformed lactobacil-

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li, (2) the bacterial supernatant from lysed transformed
lactobacilli, (3) HSV gD control, (4) the broth from non-
transformed cultures of lactobacillus, (5) bacterial
supernatants from lysed nontransformed cultures of lac-
5   tobacillus and (6) molecular weight markers. Presence of
the amylase - HSV gD fusion protein in the supernatant
from lysed transformed lactobacilli confirms that the
organisms are producing HSV gD. Presence of the amylase -
HSV gD fusion protein in the growth medium supernatant
10 confirms that the organisms are producing and _secreting_
HSV gD.

Example 3

Method of Colonization:

Six-week old female BALB/CJ mice are used since one
15 skilled in the art would appreciate that susceptibility of
younger mice to intravaginal challenge with HSV-2 appears
to vary directly with age. (Arch Virol. 93:51 (1987)).

A log-phase broth of transformed lactobacilli is
centrifuged at 800 xg. A volume of the pellet is serially
20 diluted and the number of organisms estimated by an
opacity standard. Since one skilled in the art would
appreciate that the estimated human vagina contains
approximately 10^7 organisms/ml (Rev. Inf. Dis. 134:486-
489 (1976)), at least this number of organisms is placed
25 in the mouse vagina in a volume of 0.010 ml.

Example 4

Determining Whether Colonization Has Been Accomplished:

Colonization of the mice with the transformed lac-
tobacilli is determined by scraping the wall of the vagina
30 with a calibrated loop, and immediately streaking for
colony count on agar plates with a base of LCM. Samples
are obtained every third day following colonization for 2
weeks.

The agar plates are incubated overnight at 37°C and
35 the total number of colonies counted. Lifts are made with

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nitro cellulose paper which is hybridized to a nick-
labelled HSV gD probe.   The proportion of colonies con-
taining the HSV gD insert is calculated by the number of
radiolabeled colonies per total number of colonies.   The
5   rate of persistence is computed by determining this ratio
at increasing intervals following colonization of the
animals.

Example 5
Determining An Immune Response To HSV gD:
10   The immune response to HSV gD produced by the trans-
formed colonizing bacteria is measured by testing both
blood and vaginal secretions for anti-HSV gD antibody.
Secretions are collected by flushing the vagina with 0.015
ml aliquots of TRIS buffered saline (pH 7.4) and aspirat-
15 ing. Blood specimens are collected and serum separated by
standard techniques.
        The anti-HSV gD antibody is detected by modification
of an ELISA system previously described. (J. Clin. Micro.
26:781 (1988)). Plates are coated with HSV gD or control,
20 and rinsed.   Diluted specimens (blood or vaginal secre-
tions) are added, incubated and rinsed. Anti-mouse IgA
conjugated to alkaline phosphatase is added, incubated,
and rinsed. Substrate is added and incubated, followed by
sodium hydroxide to stop the reaction.   The color
25 developed is read at 405 nm.
        Testing of the mice for anti-HSV gD antibody is
started at one week following colonization and repeated
weekly. When animals become seropositive, they are tested
until two successive samplings show no increase in
30 antibody. Then, the animals are challenged with HSV-II.
(See Example 6).

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Example 6

Virus Challenge:

When there is evidence of an immune response to HSV
gD (see Example 5), mice are challenged with HSV-2 strain
5   333, a human isolate adapted for use in mice.

A titration in unprotected mice is performed using a
vaginal inoculum of 0.01 ml containing 10^2 to 10^6 pfu of
HSV-2. Plaque titrations are done in vero cells using an
assay previously described. (J. Clin. Micro. 14:376-379
10   (1984)). A dose of HSV-2 sufficient to produce death in
50% of the unprotected animals challenged intravaginally
(i.e. one LD_{50}) is used in the challenge experiments.

The effect of the vaccine is judged by three
criteria: (1) mortality, (2) latent infection of spinal
15   ganglia, and (3) vaginal infection with HSV-2.

To test for spinal ganglia infection, bilateral
spinal ganglia are removed from the mice at three weeks
after infection. The ganglia are minced and placed
directly on a cell monolayer which is observed for c.p.e.
20   (Infec. and Immunity, 16:69-74 (1977)).

To detect vaginal infection, titers of HSV-2 in
vaginal specimens of mice colonized with the transformed
lactobacillus are compared to the control mice. Vaginal
swabs are obtained at 2 day intervals for 8 days following
25   inoculation with HSV. The swabs are placed in 1 ml of
tissue culture fluid, separated into aliquots and frozen
at-70°C. Virus is then tittered by the plaque assay
described above, and the quantity of virus in the
colonized and uncolonized control animals compared.
30   Failure to detect HSV-2 in the colonized mice indicates
that infection has been prevented.

Other and further embodiments of the invention are
readily apparent from the above description of the
invention, and these embodiments are believed to be within
35   the scope of the invention.

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Claims

1. A method of preparing a vaccine or toxoid against a pathogenic disease comprising:

a) transforming an indigenous microorganism by recombinant DNA technology to express a protective antigen;

b) growing said microorganism in a suitable culture medium; and

c) harvesting said microorganism by separating it from said culture medium.

2. A method according to claim 1 wherein the pathogenic disease is genital herpes or another sexually transmitted disease.

3. A method according to claim 1, wherein the microorganism is lactobacillus or other normal non-pathogenic inhabitants of the genital tract.

4. A method according to claim 1, where in the protective antigen is a herpes simplex virus antigen, herpes simplex virus glycoprotein D, or antigen of another sexually transmitted microorganism.

5. A vaccine or toxoid against a pathogenic disease which comprises indigenous microorganisms that have been transformed by recombinant DNA technology to express protective antigens.

6. A vaccine or toxoid according to claim 5, wherein the pathogenic disease is genital herpes or another sexually transmitted disease.

7. A vaccine or toxoid according to claim 5, wherein the microorganism is lactobacillus or other normal non-pathogenic inhabitants of the genital tract.

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8.   A vaccine or toxoid according to claim 5, wherein the protective antigen is a herpes simplex virus antigen, herpes simplex virus glycoprotein D, or antigen of another sexually transmitted microorganism.

5   9.   A vaccine against a pathogenic disease prepared by the method of claim 1.

10.   A vaccine according to claim 9, wherein the pathogenic disease is genital herpes or another sexually transmitted disease.

10   11.   A method of immunizing a host against a pathogenic disease comprising:
           a)   transforming an indigenous microorganism by recombinant DNA technology to express a protective antigen;
15   b)   culturing and harvesting said microorganism; and
           c)   colonizing an area of the host's body with said microorganisms such that the expressed protective antigens will elicit an immune response to the pathogenic
20 disease.

12.   A method according to claim 11, wherein the pathogenic disease is genital herpes or another sexually transmitted disease.

13.   A method according to claim 11, wherein the
25 indigenous microorganisms is lactobacillus or other normal non-pathogenic inhabitants of the genital tract.

14.   A method according to claim 11, wherein the protective antigen is a herpes simplex virus antigen, herpes simplex virus glycoprotein D, or antigen of another
30 sexually transmitted microorganism.

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15.   A method of preparing a vaccine against herpes simplex virus comprising:

a)   transforming lactobacillus by recombinant DNA technology to express herpes simplex virus
5   glycoprotein D;

b)   growing said lactobacillus in a suitable culture medium; and

c)   harvesting said lactobacillus by separating it from said culture medium.

10   16.   A vaccine against herpes simplex virus which comprises lactobacilli that have been transformed by recombinant DNA technology to express herpes simplex virus glycoprotein D.

17.   A method of immunizing a host against
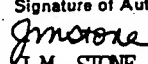15   herpes simplex virus comprising:

a)   transforming lactobacillus by recombinant DNA technology to express herpes simplex virus glycoprotein D (HSV gD);

b)   culturing   and   harvesting   said
20   lactobacillus; and

c)   colonizing the genital tract with said lactobacillus such that the expressed HSV gD will elicit an immune response to the herpes simplex virus.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04988

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.: 435/69.3, 172.3, 252.9; 424/89, 93; 935/12,65
I.P.C. (5): C12N 15/38; A61K 39/245, 39/42

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/69.3, 172.3, 252.9; 424/89,93;935/12,65

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [6]

BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,X	US,A,4,888,170 (CURTISS, III) 19 December 1989, see the entire document	1,5,11
X	Gene, volume 40, issued 1985, Amann, et.al "'ATG' vectors for regulated high-level expression of cloned genes in Escherichia coli", pages 183-190, see the entire document.	1,2,4-6,8-10
Y	Biological Abstracts, Volume 81, No. 12, issued 1986, Watanabe et al, "Protection of Mice Against herpes simplex virus infection by a Lactobacillus casei preparation (LC 9018) in combination with inactivated viral antigens", see the entire abstract, abstract no. 111724.	1-17

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
15 NOVEMBER 1990	2 5 FEB 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	J.M. STONE

Form PCT/ISA/210 (second sheet) (May 1986)

Category*	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	EP,A, 0228726, (DE VOS ET AL), 15 JULY 1987, See the entire document.	1-17

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Form PCT/ISA/210 (extra sheet) (May 1986)